REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY) February 2015	2. REPORT TYPE Open Literature	3. DATES COVERED (From - To)

4. TITLE AND SUBTITLE
Functional evaluation of biological neurotoxins in networked cultures of stem cell-derived central nervous system neurons

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hubbard, K, Beske, PH, Lyman, M, McNutt, PM

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDR-C
3100 Ricketts Point Road

Aberdeen Proving Ground, MD
21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-P14-010

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency
8725 John J. Kingman Road STOP 6201
Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

14. ABSTRACT
See reprint.

15. SUBJECT TERMS
embryonic stem cells, stem cell-derived neurons, botulinum neurotoxin detection, electrophysiology, synapse, neuronal networks, glutamatergic synapse, GABAergic synapse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Patrick McNutt
a. REPORT UNCLASSIFIED	**b. ABSTRACT** UNCLASSIFIED	**c. THIS PAGE** UNCLASSIFIED	UNLIMITED	8	**19b. TELEPHONE NUMBER** (include area code) 410-436-8044

Video Article

Functional Evaluation of Biological Neurotoxins in Networked Cultures of Stem Cell-derived Central Nervous System Neurons

Kyle Hubbard[1], Phillip Beske[1], Megan Lyman[1], Patrick McNutt[1]

[1]Research Division, Cellular Molecular Biology Branch, United States Army Medical Research Institute of Chemical Defense

Correspondence to: Kyle Hubbard at kyle.s.hubbard.ctr@mail.mil

Abstract

Therapeutic and mechanistic studies of the presynaptically targeted clostridial neurotoxins (CNTs) have been limited by the need for a scalable, cell-based model that produces functioning synapses and undergoes physiological responses to intoxication. Here we describe a simple and robust method to efficiently differentiate murine embryonic stem cells (ESCs) into defined lineages of synaptically active, networked neurons. Following an 8 day differentiation protocol, mouse embryonic stem cell-derived neurons (ESNs) rapidly express and compartmentalize neurotypic proteins, form neuronal morphologies and develop intrinsic electrical responses. By 18 days after differentiation (DIV 18), ESNs exhibit active glutamatergic and γ-aminobutyric acid (GABA)ergic synapses and emergent network behaviors characterized by an excitatory:inhibitory balance. To determine whether intoxication with CNTs functionally antagonizes synaptic neurotransmission, thereby replicating the *in vivo* pathophysiology that is responsible for clinical manifestations of botulism or tetanus, whole-cell patch clamp electrophysiology was used to quantify spontaneous miniature excitatory post-synaptic currents (mEPSCs) in ESNs exposed to tetanus neurotoxin (TeNT) or botulinum neurotoxin (BoNT) serotypes /A-/G. In all cases, ESNs exhibited near-complete loss of synaptic activity within 20 hr. Intoxicated neurons remained viable, as demonstrated by unchanged resting membrane potentials and intrinsic electrical responses. To further characterize the sensitivity of this approach, dose-dependent effects of intoxication on synaptic activity were measured 20 hr after addition of BoNT/A. Intoxication with 0.005 pM BoNT/A resulted in a significant decrement in mEPSCs, with a median inhibitory concentration (IC_{50}) of 0.013 pM. Comparisons of median doses indicate that functional measurements of synaptic inhibition are faster, more specific and more sensitive than SNARE cleavage assays or the mouse lethality assay. These data validate the use of synaptically coupled, stem cell-derived neurons for the highly specific and sensitive detection of CNTs.

Video Link

The video component of this article can be found at http://www.jove.com/video/52361/

Introduction

The overall goal of this method is to functionally evaluate synaptic activity in networked cultures of stem cell-derived neurons exposed to clostridial neurotoxins. This is the first demonstration of an *in vitro* derived model that functionally replicates the pathophysiologies responsible for clinical manifestations of botulism and validates ESNs as a suitable model for detection of CNTs, therapeutic screening and mechanistic studies.

BoNTs are highly lethal bacterial neurotoxins produced by members of the *Clostridium* species. Including the recently proposed BoNT/H, eight antigenically distinct BoNT serotypes have been described (/A-/H)[1,2]. All serotypes are expressed as 150 kDa peptides that are post-translationally nicked to produce a dichain composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond[3]. The HC mediates binding to presynaptic receptors and entry of the toxin into the neuron via synaptic endocytosis. During endosomal processing, the HC undergoes structural re-organization to form a pore in the vesicle membrane, facilitating the translocation of the LC into the presynaptic cytosol. The LC then specifically targets and cleaves soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) proteins in the presynaptic compartment: SNAP-25 (BoNT/A, /C, /E), VAMP2 (BoNT /B, /D, /F, /G) or syntaxin (BoNT/C)[1]. Cleavage of any of these SNARE proteins prevents the assembly of the synaptic exocytosis machinery, thereby blocking neurotransmitter release. This combination of efficient neuronal targeting and presynaptic localization renders BoNTs the most potent poisons known, with estimated human lethal doses as low as 0.1 - 1 ng/kg[1].

Biochemical assays can be used to detect the presence or activity of CNT LCs with high sensitivity. However, these methods fail to interrogate the ability of the toxin to bind, enter, activate and function within neurons, and therefore are indirect and possibly misleading measures of the presence of active toxin. In contrast, functional assays of intoxication such as the mouse lethality assay (MLA) comprehensively evaluate the ability of CNTs to successfully negotiate all phases of uptake and activation, providing more relevant and specific assessments of toxin activity. While the MLA is the gold-standard of BoNT detection, it is an *in vivo* method that uses death as an end-point and therefore has limited utility as a research platform. Attempts to develop cell-based models of CNT intoxication suitable for mechanistic studies and therapeutic screening have also suffered significant limitations. While primary neuron cultures offer a high degree of physiological relevance, their use is complicated by a number of factors, including high resource cost, relatively low yield, the presence of multiple neuronal subtypes and extensive regulatory

and administrative oversight involved with animal use. As an alternative to primary neurons, neurogenic cell lines (which can be induced to adopt neuron-like properties by chemical stimulation) such as neuroblastomas and adrenal chromaffin cells have been used as *in vitro* models of intoxication. Since these cells are continuously cultured prior to induction, they are highly scalable and therefore well-suited for moderate-throughput approaches. However, their relevance is questionable since induced phenotypes are typically heterogeneous, are poorly sensitive to CNTs and fail to exhibit critical neuronal behaviors, including the inability to form pre- and post-synaptic compartments that assemble into functioning synapses[4]. In the absence of physiologically intact presynaptic compartments, the full range of toxin:neuron interactions cannot be replicated and therefore functional measurements of intoxication are not possible[5]. Not surprisingly, attempts to conduct mechanistic studies or drug screening for BoNTs using induced neurogenic cell lines have resulted in findings that are inconsistent with *in vivo* and primary neuron studies[6].

It has been proposed that stem cell-derived central nervous system (CNS) neurons may provide a next-generation cell-based platform for BoNT research that combines the relevance of primary neurons with the flexibility of cultured cell lines[7-10]. In particular, mouse stem cell-derived neurons (ESNs) have been found to be highly sensitive to physiological doses of BoNT/A-/G and to replicate many *in vivo* responses to intoxication, including differential persistences, activity-enhanced intoxication, and serotype-specific potencies[5,8,11]. These behaviors suggest that *in vivo* mechanisms of BoNT uptake, processing, trafficking and activity are conserved in ESNs. However, inhibition of synaptic activity is the signature manifestation of botulism, and therefore demonstrating a loss of synaptic activity following intoxication by CNTs is essential before concluding that ESNs are a suitable *in vitro* derived cell-based model for CNT studies.

To measure the effects of intoxication with CNTs on synaptic activity, whole-cell patch-clamp electrophysiology was used to quantify monosynaptic currents in vehicle-treated or CNT-treated DIV 21[+] ESNs. We found that intoxication of ESNs with BoNT/A-/G or TeNT caused >95% loss of synaptic activity within 20 hr in all cases. Further characterization conducted 20 hr after intoxication with BoNT/A revealed a dose-dependent effect on synaptic activity, with a limit of detection below 0.005 pM and an IC_{50} value of 0.013 pM. These findings indicate that the sensitivity and time frame of electrophysiological detection of intoxication are considerably improved over comparable immunoblot-based analyses of SNAP-25 cleavage and *in vivo* mouse lethality assays, demonstrating that functional measurements of intoxication in synaptically active neuron cultures may facilitate more sensitive, specific and rapid methods to characterize the cellular response to BoNT.

Protocol

1. Adaptation of ESCs to Feeder Cell-free Suspension Culture

1. Thaw ESCs at 37 °C until a sliver of ice remains.
2. Using a P1000 pipet, gently transfer 1 - 2.5 x 10^{6} dissociated ESCs to a non-tissue culture-treated dish containing 10 ml of pre-warmed ESC medium. Incubate in a humidified tissue culture incubator for 20 hr at 37 °C and 5% CO_2.
3. After 20 hr, wash cells by gently transferring the suspension culture to a 15 ml conical tube using a 10 ml serological pipet. Pellet ESCs for 3 min at 200 x g. During spin, add 10 ml of fresh ESC medium back to low-adhesion dish.
4. Aspirate supernatant, taking care to avoid dislodging the cell pellet, and add 1 ml fresh ESC medium to cell pellet. Transfer cells to the bacterial dish using a P1000 pipet and return to the incubator.
5. Observe cells daily until aggregates become visible to the naked eye (typically 4 - 8 days (d); aggregates will be 0.2 - 0.5 mm). If aggregates are not apparent after 4 d, repeat step 1.3. Once aggregates are visible, dissociate and maintain ESCs as described in Section 2.

2. ESC Passaging and Maintenance

1. Transfer ESC aggregates to a 15 ml conical tube using a sterile 10 ml pipet and allow aggregates to settle to a compact pellet (typically 3 - 5 min). Aspirate the supernatant, being careful to avoid disrupting the cell pellet, and wash aggregates with 5 ml PBS. Although gravity settling is recommended, alternatively pellet cells at 100 x g for 2.5 min instead to save time.
2. Aspirate PBS and add 500 μl trypsin. Incubate aggregates in trypsin for 3 min in a water bath at 37 °C. Add 500 μl ESC medium to dilute the trypsin and gently triturate 10 times with a P1000 pipet to break up aggregates. Count cells using a hemocytometer.
3. Pellet dissociated cells for 3 min at 200 x g and resuspend cells to a final concentration of 1.0 x 10^{7} cells/ml in ESC medium. Transfer 150 μl (1.5 x 10^{6} cells) to 10 ml of fresh ESC medium in a 10 cm bacterial dish and return to tissue culture incubator for 48 hr. Excess ESCs can be differentiated, cryopreserved at 1 - 5 x 10^{6} cells/ml in 90% ESC medium supplemented with 10% DMSO, or discarded.
4. Passage aggregates every 48 hr. Aggregates ready for passage will be clearly visible, with diameters exceeding 0.5 mm.
   NOTE: Thawing and culture of cryopreserved, suspension-adapted cells are accomplished per steps 1.2 - 1.4. Aggregates will be ready for passaging within 48 - 72 hr.

3. Neuronal Differentiation

NOTE: Conduct steps 3.1 - 3.5 prior to noon and step 3.7 after noon. An overview of the differentiation procedures is presented in **Figure 1A**.

1. Dissociate ESCs as in steps 2.1 - 2.5. Transfer 350 μl (3.5 x 10^{6}) of dissociated ESCs to a 10 cm low-attachment dish containing 25 ml differentiation medium. Place on an orbital shaker set to 30 - 45 rpm inside a tissue culture incubator at 37 °C with 5% CO_2. This is the first day of differentiation, termed day *in vitro* (DIV) -8.
   NOTE: The use of an ultra-low attachment dish increases the cost of the method, but produces slightly larger yields than bacterial Petri dishes since aggregates can occasionally adhere to Petri dishes. If different dish sizes are preferred, medium volumes and cell numbers can be scaled accordingly.
2. After 48 hr (DIV -6), use a 25 ml pipet to transfer the differentiating cell aggregates to a 50 ml conical tube. Immediately add a fresh 25 ml of differentiation medium to the Petri dish.

3. Allow aggregates to settle over 2 - 5 min, producing a visible pellet that is 1 - 2 mm deep. Ignore single cells or small aggregates remaining in suspension. Carefully aspirate the medium and transfer the cell pellet back to the petri dish using a P1000. Place on rotary shaker in a tissue culture incubator.
4. At DIV -4 repeat step 3.2. The pellet will be 2 - 4 mm deep. Replace 30 ml differentiation medium supplemented with 6 µM all-trans retinoic acid (RA) to the petri dish. Return to rotary shaker in the tissue culture incubator for an additional 48 hr.
5. At DIV -2 repeat step 3.3. The pellet will be 4 - 8 mm deep at this point.
6. At DIV -1, prepare plating surfaces as in Section 4.
7. At DIV 0, thaw 5 ml of pre-aliquoted and frozen NPC trypsinization medium at 37 °C for 5 - 10 min and place in a tissue culture hood. Using a 25 ml pipet, transfer differentiating aggregates to a 50 ml conical tube. Allow aggregates to settle and carefully aspirate medium. Wash pellet twice with 10 ml PBS, allowing the aggregates to settle between washes.
8. After the second PBS wash, add 5 ml of NPC trypsinization medium to the pellet and incubate at 37 °C for 5 min. Gently flick the tube after 2.5 min.
9. Add 5 ml of 0.1% soybean trypsin inhibitor (STI) to inactivate trypsin and mix by inverting. Gently triturate 10 - 15 times with a 10 ml serological pipet until a relatively homogenous cell suspension is produced.
10. Slowly transfer the cell suspension to a 40 µm or 70 µm cell strainer placed in the top of a 50 ml conical tube. Once all the suspension has been filtered, add 1 ml of N2 medium to wash remaining cells through the filter and pellet the dissociated cell suspension for 6 min at 200 x g.
11. Aspirate medium without disturbing the pellet. Wash cells twice with 10 ml N2 medium, pelleting cells for 5 min at 200 x g and triturating between washes with a P1000. Prior to the second wash, count cells using a hemocytometer.
12. Resuspend cells in N2 medium at 1×10^7 cells per ml and plate ESNs at a cell density of 150,000 - 200,000 cells/cm^2.
13. Transfer newly plated ESNs to a humidified tissue culture incubator at 37 °C and 5% CO_2 and maintain as in Section 5.

4. Preparing Culture Surfaces for Plating Neural Precursors at DIV 0

1. Prepare tissue culture-treated dishes at least 1 day prior to plating. Add sufficient polyethyleneimine (PEI; 25 µg/ml in sterile H_2O) or poly-D-lysine (PDL; 100 µg/ml in sterile H_2O) to cover tissue-culture-treated plastic dishes and incubate O/N at 37 °C.
2. The morning of plating, wash dishes twice with double-distilled H_2O and once with PBS. After the final wash, add sufficient N2 medium to cover the dish (*e.g.,* 1 ml per well of a 12-well dish or 4 ml per 6 cm dish).
3. Prepare 18 mm glass coverslips at least one day prior to neuron plating. Clean coverslips by plasma-cleaning for 4 min.
4. Immediately transfer cleaned coverslips to an ethanol-washed parafilm in the bottom of a large sterile dish and add 400 µl of PEI or PDL solution, prepared as in step 4.1. Incubate O/N at 37 °C in a tissue culture incubator.
5. In the morning, wash coverslips three times with water and add 5 µg/ml laminin in PBS for 1 - 3 hr at 37 °C. Prior to dissociating neurons, aspirate the laminin and immediately transfer the coverslip to a well of a 12-well dish containing 1 ml of NPC medium, being sure to keep the treated side facing up.
6. Store dishes and coverslips at 37 °C until NPCs are ready to plate.

5. Maintenance of Neurons

1. At DIV 1, aspirate medium and replace with N2 culture medium.
2. At DIV 2 and 4, aspirate medium and replace with B27 culture medium.
3. At DIV 8, aspirate medium and replace with B27 culture medium containing mitotic inhibitors to eliminate contaminating non-neuronal cells.
4. At DIV 12, replace with B27 culture medium.
5. Do not remove DIV 12$^+$ ESNs from 5% CO_2 until ready to use.

6. Measured Inhibition of Synaptic Transmission (MIST) Assay for Quantifying Miniature Excitatory Post-synaptic Currents

CAUTION: The Clostridial neurotoxins are the most poisonous substances known, with estimated human LD$_{50}$ values as low as 0.1 - 1 ng/kg. Obtain necessary approvals prior to using these toxins and use appropriate precautions.

1. Carefully dilute BoNT/A to 100x the desired final concentration in ESN culture medium and warm to 37 °C. Add appropriate volume of toxin to DIV 21$^+$ ESN cultures, swirl the culture dish, and return to incubator.
2. If cells will be analyzed more than 4 hr after intoxication, add toxin to the dish and swirl without removing from 5% CO_2, such as directly in the incubator or in a constant CO_2 chamber.
3. At appropriate time point, aspirate ESN culture medium and wash twice with extracellular recording buffer (ERB). Add 4 ml of ERB supplemented with 5.0 µM tetrodotoxin and 10 µM bicuculline, blocking action potentials and antagonizing GABA$_A$ receptor activity, respectively.
4. Transfer dish to electrophysiology rig. Neither perfusion nor temperature control is required for the MIST assay.
5. Pull borosilicate glass using a micropipette puller to produce a recording pipette with 5-10 mΩ of resistance and fill with intracellular recording buffer. Gently dip filled recording pipette in siliconizing reagent prior to recording.
6. Using an air-filled syringe, provide positive pressure as the recording pipette is lowered into the ERB. After gently landing the recording pipette on the soma of the neuron to be recorded, remove positive pressure and form a gigaseal. Decrease the holding voltage to -70 mV. Carefully apply negative pressure to break into whole-cell configuration.
7. Switch to current-clamp to monitor and record resting membrane potential. Without adjustment for liquid junction potentials, the resting membrane potential will be between -67 to -82 mV.
8. Perform a continuous -70 mV voltage-clamp recording for 4 - 5 min to detect miniature excitatory post-synaptic currents (mEPSCs).
9. Analyze 4 min of recorded data for mEPSC detection using spike detection software with the following settings: Threshold, 5; Period to search a local maximum, 10,000 µsec; Time before a peak for baseline, 5,000 µsec; Period to search a decay time, 20,000 µsec; Fraction of

peak to find a decay time, 0.37; Period to average a baseline, 1,000 µsec; Area threshold, 20; Number of points to average peak, 1; Direction of peak, negative.

10. Collect and save information on detected events. Divide the number of detected events in 4 min by 240 to determine the mEPSC frequency in Hz.
11. Collect mEPSC frequencies for 8 - 12 controls and 8 - 12 BoNT-treated samples for each exposure condition. Analyze frequency against age- and lot-matched controls. Determine the statistical significance of % inhibition of synaptic activity using one-way ANOVA testing and Dunnett's *post-hoc* test.

Representative Results

We have developed a differentiation protocol that enables the economic production of large quantities of highly pure stem cell-derived neurons from mouse ESCs (detailed in **Figure 1A**)[8,11]. This method has been used over a period of years to reproducibly differentiate privately generated and commercially available ESC lines into neurons of defined lineages[12-14]. Critical elements of this protocol include (1) adaptation of ESCs to feeder cell-free suspension culture; (2) proper maintenance of suspension cultures; and (3) differentiation under rotary conditions. Transition to suspension culture dramatically reduces the time and cost of ESC maintenance, and obviates the need to remove feeder cells prior to differentiation. Following suspension adaptation, Oct3/4 expression is consistent through at least 30 passages, indicating that suspension adaptation does not alter expression of pluripotency markers (**Figure 1B-D**). ESCs are suitable for neuronal differentiation once cell yield consistently exceeds 1×10^7 cells per passage. This typically occurs within ten passages after suspension adaptation or five passages after thawing of ESCs that were previously suspension-adapted. Mechanical rotation of differentiating aggregates was also found to be critical to increased neuronal yield. The addition of a rotary shaker eliminated super-aggregate formation, increasing aggregate viability and producing a ~300% increase in the yield of neural progenitor cells (NPCs) at DIV 0 (**Figure 1E**).

A typical differentiation starts with 3.5×10^6 ESCs at DIV -8 and produces 115×10^6 NPCs at DIV 0, roughly 60% of which will survive and become neurons. The remaining 40% comprise non-neuronal cells and are largely eliminated by serum deprivation between DIV 0 - 1. The small number of persisting glia can be removed by addition of mitotic inhibitors from DIV 2 - 4 or DIV 8 - 12. Plating density is critical at DIV 0; neurons plated too sparsely will not survive beyond 2 weeks. Within days of plating, differentiated neurons exhibit neuronal morphologies and compartmentalize neurotypic proteins, such as the somatodendritic marker MAP2 and the axonal marker Tau (**Figure 2A**). By DIV 14 synapsin-1[+] puncta can be identified at axodendritic interfaces, suggestive of synapse formation. This is consistent with the expression of synaptic marker proteins prior to DIV 7 [8,11]. Neuronal morphologies continue to mature through DIV 21, at which time cultures exhibit elaborate axodendritic arbors and large synaptic puncta (**Figure 2A**). If maintained appropriately, ESNs remain viable and active for at least 4 weeks after plating.

Longitudinal expression profiling using RNA-sequencing corroborated morphological and proteomic evidence of neuronal specification and maturation[11,15]. Representative markers of developmental progression exhibited stage-specific expression profiles, including Oct3/4, Nestin, DCX, NeuN and KCC2 (**Figure 2B**). By DIV 0, ESNs expressed abundant copies of neuron-specific structural proteins, including MAP2 and Tau. Consistent with previous findings, markers for only two neuronal subtypes were observed: vGluT2-expressing midbrain/hindbrain glutamatergic neurons and GABAergic interneurons[8]. Correspondingly, a wide array of glutamatergic and GABAergic markers exhibited sharp increases in expression between DIV 0 and DIV 7, including pre-synaptic SNARE proteins required for neurotransmitter release; neurotransmitter receptors required for post-synaptic responses; and scaffolding proteins required to tether these receptors to the post-synaptic membrane. Neurons exhibit mature intrinsic electrical characteristics by DIV 14 and spontaneous miniature excitatory post-synaptic currents by DIV 16 [16].

The Measured Inhibition of Synaptic Transmission (MIST) assay was used to evaluate the effect of intoxication on synaptic activity. By comparing mEPSC frequencies between intoxicated and vehicle-treated DIV 24[+] ESNs, MIST provides a quantitative and specific measurement of intoxication based on the functional inhibition of synaptic activity (**Figure 3A**). MIST was used to measure the effects of BoNT/A-/G or TeNT on synaptic activity in ESNs at 20 hr after bath addition of each toxin. Toxins were added at a concentration equivalent to 10-fold the EC_{50} value, as previously determined by immunoblot analysis of SNARE protein cleavage[11]. All toxins reduced mEPSC frequencies to less than 5% of vehicle-treated controls. Reductions in synaptic rates were not attributable to cell death or altered intrinsic responses, since intoxicated ESNs were capable of being patched, fired repeated action potentials in response to current injection and exhibited no significant alteration in resting membrane potential (**Figure 3B,C**).

To compare the sensitivity of MIST to existing methods to detect CNTs, the limit of detection and median inhibitory concentration (IC_{50}) were determined 20 hr after addition of BoNT/A to ESNs. Intoxication by as little as 0.005 pM BoNT/A produced a statistically significant reduction in mEPSC frequency, with an IC_{50} value of 0.013 pM and complete silencing of synaptic activity above 0.5 pM (**Figure 4A**). This IC_{50} value corresponds to approximately 0.5 mouse lethal units/ml, suggesting that MIST is twice as sensitive and 2- to 4-fold faster than the MLA in detecting the presence of BoNT/A. Immunoblot measurements of SNAP-25 cleavage produced an EC_{50} value of 0.38 pM and a minimum detectable dose of 0.05 pM, indicating that MIST is approximately 30-fold more sensitive than immunoblot-based detection of cleaved SNARE proteins (**Figure 4B**).

Figure 1. Suspension-adapted ESNs remain mitotically stable and express markers of pluripotency. (**A**) Schematic of ESC maintenance and differentiation. The presence or absence of retinoic acid (RA) or leukemia inhibitory factor (LIF) is marked by a + or –. A comparison between days *in vitro* (DIV) and classical developmental stages (DS) for primary neuron cultures is provided[17]. (**B**) Proliferation rates for R1, D3 and C57BL/6J ES cell lines stabilize by five passages after transition to suspension culture. (**C**) Flow cytometry data demonstrate no substantive change in Oct3/4 expression in the R1, D3 and C57BL/6J ES cell lines measured over 25 passages in suspension culture (n = 6 for each). (**D**) Actual cell yields during routine passaging for a suspension-adapted R1 ESC line measured between passages 5 and 30 (black line). Theoretical cumulative yields if no cells are discarded during passaging are also presented (red line). (**E**) Bright-field images of DIV 0 aggregates produced under static (left) or rotary conditions (right). Rotary conditions produced spherical aggregates without agglomeration and increased NPC yields 3-fold (p < 0.001, determined using Student's t-test)[11]. * indicates a *P* < 0.05. This figure has been modified from Hubbard *et al.*[11] Please click here to view a larger version of this figure.

Figure 2. Morphological, proteomic and transcriptomic evidence of neuronal specification and maturation. (**A**) Immunocytochemistry from DIV 7 – DIV 49 demonstrates neuronal arborization and the appearance of synaptic puncta at axodendritic interfaces. Axons are labeled with Tau (green), dendrites are labeled with MAP2 (red), pre-synaptic compartments are labeled with synapsin (white), and nuclei are stained with DAPI (blue). (**B**) Longitudinal expression profiling of representative genes demonstrating developmental stage-specific expression and specifying neuronal subtypes. All gene transcripts are expressed as approximate number of transcripts per cell. Please click here to view a larger version of this figure.

Figure 3. ESNs undergo inhibition of synaptic activity when exposed to BoNT/A-G and TeNT. (**A**) Representative traces from ESNs collected 20 hr after bath addition of ~10 x EC_{50} values of BoNT/A - /G, TeNT or vehicle. Each neurotoxin reduced synaptic activity by over 95% in comparison to controls. (**B**) Intoxicated neurons remained able to fire repeated APs in response to depolarizing current injection (as demonstrated for BoNT/A, but observed in all intoxicated cultures) and (**C**) exhibited no changes in the negative resting membrane potential (C; n > 18 for each treatment). Please click here to view a larger version of this figure.

Figure 4. Determination of the sensitivity of MIST in BoNT/A-treated ESNs. (**A**) MIST measurements of synaptic activity 20 hr after bath addition of BoNT/A. Representative voltage-clamp trace segments exhibited a decrease in mEPSC frequency following BoNT/A exposure (left side). Quantitation of mEPSC frequency (bar graph, right side, n = 20 for controls; n = 11 - 22 for each dose) confirms a dose-dependent decrease in mEPSC frequency. The median inhibitory concentration was determined with a least-squares fit of a non-linear regression using a four parameter variable slope (R^2 > 0.91). Note the limit of detection by MIST in ESNs is at least 0.005 pM. (**B**) BoNT/A intoxication of ESNs results in cleavage of SNAP-25 as visualized by gel mobility shifts (a representative western blot on the left side with a bar graph showing quantification of SNAP-25 cleavage on the right; n = 4). Note the decreased sensitivity using SNARE protein cleavage (SNAP-25) as an endpoint (EC_{50} of 0.36 pM) *vs.* MIST (IC_{50} of 0.013 pM). * indicates a *P* < 0.05; *** indicates a *P* < 0.001. Please click here to view a larger version of this figure.

Discussion

The current gold standard for CNT detection, serotype determination and quantitation is the MLA. The MLA interrogates the entire range of host:toxin interactions required for intoxication to occur *in vivo* (*e.g.,* toxin binding to a cell surface receptor, internalization of the toxin-receptor complex, LC translocation into the cytoplasm, LC-mediated cleavage of substrate and inhibition of synaptic neurotransmission)[18]. However, although the MLA offers a physiologically relevant model of intoxication, it is resource intensive, can be confounded by contaminants and involves large numbers of mice with death as an endpoint. Alternatively, cell-based assays for BoNT detection and quantitation have been limited to primary neuron cultures, which also require animal use, or to neuroblastoma cell lines, which fail to form synapses and typically exhibit poor sensitivity to neurotoxins[8]. To date, most measurements of intoxication in these cell-based platforms have relied on detection of the proteolytic cleavage of SNAP-25, VAMP1/2 or syntaxin-1 [11]. This is problematic because SNARE protein cleavage has been shown to be non-linearly associated with synaptic inhibition *in vivo* and therefore may not accurately represent intoxication or recovery from intoxication[19,20].

An ideal cell-based model system for CNT detection would (i) be neuron-based; (ii) be synaptically coupled with neurotypic electrical responses; (iii) be highly sensitive to all CNT serotypes or subtypes; and (iv) offer scalability, throughput and assay times that are equivalent to or improved over the MLA, at lower cost, and without requiring animal use. To meet these requirements, we developed a method to produce large quantities of highly enriched, networked cultures of glutamatergic and GABAergic neurons from commercially available mouse ESC lines. We then developed the MIST assay to quantify synaptic inhibition in response to intoxication with TeNT and BoNT/A-/G. While the MIST assay can be conducted on any synaptically active neuron population (*e.g.,* primary neurons or brain slices), currently ESNs are the only *in vitro* derived neuron model that reproducibly develops emergent network behaviors and is therefore appropriate for the MIST assay.

Producing sufficient quantities of neurons of defined lineage for CNT studies required several innovations in ESC culture and differentiation. First, by selecting for and culturing ESCs that can survive suspension-adaptation, we eliminated the potential for contamination by feeder cells and the need to purify feeder cells from ESCs at the start of differentiation. Although the molecular mechanisms underlying suspension-adaptation are unknown, suspension-adapted ESCs remain mitotically active, retain Oct3/4 expression and continue to be neurogenic. Using this method, ~1.5 x 10^7 ESCs are typically recovered each passage. Second, the production of NPCs was increased ~300% by incorporating mechanical agitation to prevent agglomeration during differentiation, ostensibly increasing nutrient accessibility to the interiors of aggregates. During the process, we found that the serum used for ESC culture and neuronal differentiation is the most critical component in maintaining neurogenic ESCs. For best success, we recommend suspension adapting ES cells to each lot of serum and stockpiling serum at -20 °C to support ESC culture and neuronal differentiation through the expiration date.

As with most synaptically active cultures, DIV 14^+ ESNs are highly susceptible to abrupt changes in pH, and even a brief exposure to atmospheric CO_2 concentrations can result in neurotoxicity within 24 hr. For experiments requiring treatment of cultures followed by incubations lasting O/N or longer, neurons should be treated directly in the incubator or transferred to a constant CO_2 chamber prior to experimentation.

A variety of techniques confirmed neurogenesis and neuronal maturation, including ICC, transcriptional profiling and whole-cell patch-clamp electrophysiology. Temporal changes in gene expression and neuron morphology were consistent with a rapid progression through the developmental stages of neurogenesis, and by DIV 16, ESNs exhibited excitatory and inhibitory miniature post-synaptic currents with emergent network behaviors[16]. Evidence of synaptic activity suggested that ESNs may replicate the pathophysiologies responsible for the clinical manifestations of botulism and tetanus. This was confirmed by using MIST to show that intoxication with BoNT/A-/G or TeNT impaired mEPSC frequencies by >95% compared to vehicle-treated or untreated neurons.

Measurements of the sensitivity of MIST for BoNT/A indicated a median inhibitory concentration (IC_{50}) of 0.013 pM (equivalent to 0.5 mouse lethal units/ml) and a limit-of-detection below 0.005 pM, measured at 20 hr after bath addition. Based on these values, MIST is approximately twice as sensitive as and 2 - 4 times faster than the MLA and 30-fold more sensitive than immunoblot-based detection of cleaved SNAP-25.

Collectively, these data suggest that networked populations of stem cell-derived neurons offer a physiologically relevant, cell-based model of intoxication. In combination with improved methods to derive networked ESN cultures from suspension-adapted ESCs, the use of MIST should reduce the need for animal testing and the associated disadvantages, costs and ethical concerns of the MLA while providing a more rapid, sensitive and specific measure of intoxication. Although whole-cell patch-clamp electrophysiology is a low-throughput method for identifying the presence of active neurotoxins, it offers a resolution and speed that is unachievable using molecular methods. These findings also provide evidence that the application of activity-dependent methods to evaluate synaptic activity and network behavior may enable the rapid and specific detection of neurotoxins. Such higher-throughput approaches would make mechanistic studies, therapeutic screening or diagnostic assays feasible for a wide array of neuromodulatory agents, including the CNTs.

Disclosures

The U.S. Government has filed two international patent applications that incorporate methods described in this article: PCT/US2014/22042, titled "Methods of detecting neurotoxin using synaptic activity"; and PCT/US13/40641, titled "Toxin detection using stem cell-derived neurons."

Acknowledgements

References

1. Simpson, L. L. Identification of the major steps in botulinum toxin action. *Annual Review Of Pharmacology And Toxicology.* **44**, 167-193 (2004).
2. Dover, N., Barash, J. R., Hill, K. K., Xie, G., Arnon, S. S. Molecular Characterization of a Novel Botulinum Neurotoxin Type H Gene. *The Journal Of Infectious Diseases.* (2013).
3. Singh, B. R. Botulinum neurotoxin structure, engineering, and novel cellular trafficking and targeting. *Neurotoxicity Research.* **9**, 73-92 (2006).
4. Larsen, J. C. U. S. Army botulinum neurotoxin (BoNT) medical therapeutics research program: past accomplishments and future directions. *Drug Development Research.* **70**, 266-278 (2009).
5. McNutt, P., Celver, J., Hamilton, T., Mesngon, M. Embryonic stem cell-derived neurons are a novel, highly sensitive tissue culture platform for botulinum research. *Biochem Bioph Res Co.* **405**, 85-90 (2011).
6. Eubanks, L. M., *et al.* An in vitro and in vivo disconnect uncovered through high-throughput identification of botulinum neurotoxin A antagonists. *Proceedings of the National Academy of Sciences of the United States of America.* **104**, 2602-2607 (2007).
7. Pellett, S., *et al.* Sensitive and quantitative detection of botulinum neurotoxin in neurons derived from mouse embryonic stem cells. *Biochem Biophys Res Commun.* **404**, 388-392 (2011).
8. Mesngon, M., McNutt, P. Alpha-Latrotoxin Rescues SNAP-25 from BoNT/A-Mediated Proteolysis in Embryonic Stem Cell-Derived Neurons. *Toxins.* **3**, 489-503 (2011).
9. Kiris, E., *et al.* Embryonic stem cell-derived motoneurons provide a highly sensitive cell culture model for botulinum neurotoxin studies, with implications for high-throughput drug discovery. *Stem Cell Res.* **6**, 195-205 (2011).
10. Whitemarsh, R. C., *et al.* Novel application of human neurons derived from induced pluripotent stem cells for highly sensitive botulinum neurotoxin detection. *Toxicological Sciences.* **126**, 426-435 (2012).
11. Hubbard, K. S., *et al.* High yield derivation of enriched glutamatergic neurons from suspension-cultured mouse ESCs for neurotoxicology research. *BMC Neurosci.* **13**, 127 (2012).
12. Brook, F. A., Gardner, R. L. The origin and efficient derivation of embryonic stem cells in the mouse. *Proceedings of the National Academy of Sciences of the United States of America.* **94**, 5709-5712 (1997).
13. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., Kemler, R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *Journal Of Embryology And Experimental Morphology.* **87**, 27-45 (1985).
14. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., Roder, J. C. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America.* **90**, 8424-8428 (1993).
15. Hubbard, K., Gut, I. M., Lyman, M. E., McNutt, P. M. Longitudinal RNA sequencing of the deep transcriptome during neurogenesis of cortical glutamatergic neurons from murine ESCs. *F1000Research.* **2**, (35), (2013).
16. Gut, I. M., *et al.* Novel application of stem cell-derived neurons to evaluate the time- and dose-dependent progression of excitotoxic injury. *PLoS One.* **8**, e64423 (2013).
17. Dotti, C. G., Sullivan, C. A., Banker, G. A. The establishment of polarity by hippocampal neurons in culture. *The Journal Of Neuroscience : The Official Journal Of The Society For Neuroscience.* **8**, 1454-1468 (1988).
18. Capek, P., Dickerson, T. J. Sensing the deadliest toxin: technologies for botulinum neurotoxin detection. *Toxins.* **2**, 24-53 (2010).
19. Raciborska, D. A., Trimble, W. S., Charlton, M. P. Presynaptic protein interactions in vivo: evidence from botulinum A, C, D and E action at frog neuromuscular junction. *The European Journal Of Neuroscience.* **10**, 2617-2628 (1998).

20. Baskaran, P., Thyagarajan, B. Acute and chronic effects of botulinum neurotoxin a on the mammalian neuromuscular junction. *Muscle & Nerve.* **50**, 206-215 (2014).